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RNA-mediated gene activation

Alan L Jiao and Frank J Slack*

Department of Molecular, Cellular and Developmental Biology; Yale University; New Haven, CT USA

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Abbreviations: AGO, Argonaute; BDNF, brain-derived neurotrophic factor; hnRNP, heterogeneous nuclear ribonuclear protein; Hox, homeobox; IFNg, interferon gamma; IL, interleukin; lincRNA, long intergenic non-coding RNA; lncRNA, long non-coding RNA; miRNA, microRNA; NAT, natural antisense transcript; ncRNA, non-coding RNA; nt, nucleotide; PAWR, PRKC apoptosis WT1 regulator; PcG, Polycomb group; piRNA, PIWI-interacting RNA; PRC2, polycomb repressive complex 2; PRE, polycomb responsive element; RNAa, RNA activation; RNAi, RNA interference; RNAP II, RNA polymerase II; saRNA, small activating RNA; siRNA, small interfering RNA; TRE, trithorax responsive element; trxG, trithorax group; TSS, transcriptional start site; Ubx, Ultrabithorax

The regulation of gene expression by non-coding RNAs (ncRNAs) has become a new paradigm in biology. RNA-mediated gene silencing pathways have been studied extensively, revealing diverse epigenetic and posttranscriptional mechanisms. In contrast, the roles of ncRNAs in activating gene expression remains poorly understood. In this review, we summarize the current knowledge of gene activation by small RNAs, long non-coding RNAs, and enhancer-derived RNAs, with an emphasis on epigenetic mechanisms.

Introduction

In the past decade, high throughput genomic technologies have revealed that the majority of the mammalian genome is transcribed.[1,2] The production of this diverse array of RNA molecules is often spatially and/or temporally regulated, and includes RNA species of various sizes that can overlap gene promoters, enhancers, exons and introns, on both sense and antisense strands. As only a tiny fraction of the transcriptome encodes for protein, the question of whether and how the remaining mass of ncRNAs serve biological functions is currently debated.[2-4] One theory is that some of these RNAs can help direct epigenetic modifications to specific genomic loci, both in *cis* and in *trans*.[5,6] This largely stems from some of the well-studied long ncRNAs, such as *Xist* and *Air,* which have been shown to epigenetically silence gene expression.[7-9] Many smaller ncRNAs have also been heavily implicated in gene silencing, both epigenetically and posttranscriptionally.[10]

Amidst the thousands of reports on gene regulation by ncRNAs, only a handful of cases have described an RNA-mediated gene activating role. In addition, some of these cases have been attributed to indirect effects of gene silencing pathways.[11] However, taken together with a number of reports that have emerged in the last few years, it appears that ncRNAs can indeed directly upregulate gene expression through a variety of mechanisms. Although many of these are poorly understood, a major theme has been the association of activating epigenetic marks at many ncRNA-targeted genomic sites.

In this review, we discuss the current understanding of ncRNA-mediated gene activation. We begin with a brief outline of small RNAs and their gene silencing roles. We discuss the serendipitous discovery of small activating RNAs (saRNAs), and summarize the progress of the field since. We then move onto the gene activating roles of longer ncRNAs, emphasizing epigenetic mechanisms and common regulatory themes.

*Correspondence to: Frank J Slack; Email: frank.slack@yale.edu
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Small RNAs and Gene Silencing

The most well understood small ncRNAs have been classified into three major categories, all of which mediate gene repression: microRNAs (miRNAs), small interfering RNAs (siRNAs), and PIWI-interacting RNAs (piRNAs).[10,12,13] The mature forms of these RNAs are 20–30 nucleotide (nt) molecules that associate with a member of the Argonaute (AGO) superfamily of proteins, the central effectors of RNA interference (RNAi) pathways.[14-16] miRNAs and siRNAs are typically known as posttranscriptional gene silencers, guiding AGO complexes to complementary mRNAs in the cytoplasm, inducing transcript degradation and blocking translation.[12,15] piRNAs associate with the PIWI clade of Argonautes to silence transposons in the germline and are required for fertility in many organisms.[17,18]

Although much of the initial work on small RNA pathways has been focused toward gene silencing mechanisms in the cytoplasm, it has become clear that many of these RNAs could also epigenetically silence transcription in the nucleus.[19-23] AGO proteins were once again shown to be the key factors in mediating this nuclear RNAi, demonstrating their importance and multiple functions as gene silencing complexes. The RNA-induced transcriptional silencing complex, consisting mainly of a small RNA-loaded AGO protein, is also able to mediate co-transcriptional gene silencing.[24,25]

Gene Activation by Small RNAs

In an attempt to use small RNAs to silence gene transcription in human cells, Li et al.[26] noticed a set of promoter-targeting duplex RNAs that actually resulted in the specific activation of the targeted genes. They designed a series of 21 bp RNA molecules corresponding to the promoter regions of three genes: *E-cadherin, p21,* and *VEGF*. Surprisingly, an induction in gene expression resulted in all three cases. This transcriptional activation was dependent on the Ago2 protein, and correlated with a loss of H3K9 promoter methylation.[26] The authors described this phenomenon as "RNA activation," or RNAa, as it involved both small duplex RNAs and an AGO complex.

Since then, multiple studies have been conducted testing the general applicability and design principles of the small RNAs used for gene activation. Progress has also been made toward understanding the molecular targets of these RNAs, their associated protein factors, and their epigenetic influence at complementary genomic loci.

Small activating RNAs (saRNAs): general applicability and targeting principles

In addition to *E-cadherin, p21*, and *VEGF*, a large number of other genes, including the progesterone receptor (*PR*), *p53*, and *Nanog*, have since been shown to be susceptible to RNAa.[27-29] Moreover, RNAa appears to be a widespread phenomenon that is conserved at least in mammals, as saRNAs have been reported to function in mouse, rat, and nonhuman primate cells.[28] However, the successful design of saRNAs remains a hit-or-miss process. First off, the effects of a given saRNA can be cell-type specific. Further, RNAa is extremely sensitive to the positioning of the saRNA target site relative to the transcriptional start site (TSS). While some studies suggest that targeting 200 bp–1200 bp upstream of the TSS may be optimal, others have successfully used saRNAs that actually overlap the TSS.[28,30] Yue et al.[31] has even shown the transcriptional activation of the PR gene by duplex RNAs targeting downstream of its 3′UTR.

The epigenetic state of the underlying chromatin may also help determine saRNA efficacy. For instance, the *E-Cadherin* gene is silenced in HeLa cells due to DNA hypermethylation around its promoter.[26] This hypermethylation was shown to prevent saRNA-mediated E-cadherin activation. Interestingly, co-treatment of cells with saRNAs and 5-azacytidine, a DNA demethylating agent, elevated levels of E-cadherin significantly more than 5-azacytidine treatment alone.[26] It remains to be seen if the inhibition of RNAa by DNA methylation is a general phenomenon.

DNA and non-coding transcripts as saRNA targets

Recent evidence of low level transcription throughout the genome—including through promoter regions—implies that saRNAs may base-pair either with nascent transcripts, or with the promoter DNA. A report by Schwartz et al.[27] suggests that nascent transcripts are the more likely target of saRNAs. They showed that the specific degradation of an antisense transcript across the *PR* promoter could reverse saRNA-mediated gene activation. Furthermore, pull-down of biotinylated saRNAs revealed a physical association between the sense saRNA strand and the antisense promoter transcript. PR promoter DNA was not detected in the pull-down, suggesting a lack of direct interaction between saRNAs and chromatin. This is in contrast to a study by Hu et al.[32] at the *p21* gene promoter, where they showed that both biotinylated strands of the saRNA duplex interacted with promoter DNA. The limited anecdotal evidence currently available makes it difficult for any general mechanisms to be established.

Associated proteins and epigenetic effects of saRNAs

Right at the outset, kinetic differences between classical RNAi and RNAa suggested that epigenetic changes may play a more critical role in the latter. While the effects of posttranscriptional gene silencing mediated by siRNAs can be seen within hours of transfection, gene activation by saRNAs is typically delayed 24–48 h.[33] A model whereby saRNAs mediate transcriptional upregulation through epigenetic means could account for its delayed kinetics. The fact that a single saRNA transfection can maintain gene upregulation for almost two weeks also supports this notion.[33] Below we discuss the specific epigenetic mechanisms by which small RNAs and their protein cofactors have been observed to activate gene expression.

RNAa is almost always accompanied by changes in histone modifications around the target promoter. Trimethylated H3K4 (H3K4me3), a classic marker of active transcription, was shown to increase at the *PR* and *cyclin B1* promoters following induction by their respective saRNAs.[28,30] Interestingly, treating cells with a protein methyltransferase inhibitor reduced the saRNA-mediated PR gene activation back to near basal levels. Inhibiting histone deacetylation was also shown to reverse RNAa of the PR gene, suggesting that both histone methylation and deacetylation were required for gene activation.[30]

Perhaps not surprisingly, the specific histone changes that are known to occur following saRNA treatment differ for the various genes and cell types examined. For instance, saRNAs targeting *PRKC apoptosis WT1 regulator* (*PAWR*), *PR*, and *interleukin (IL)-24* promoters have all been shown to increase dimethylated H3K4 (H3K4me2), also a marker of active transcription.[30,34,35] However, H3K4me2 was not enriched upon saRNA treatments at either the *E-cadherin* or *IL-32* gene promoters.[26,35] A reduction in H3K27me3 (typically associated with transcriptional silencing) was associated with the saRNA induction of *PR*, but not of the *LDL* receptor.[31,36] Intriguingly, the reduction of H3K27me3 at the *PR* promoter was induced by an saRNA targeted just beyond the *PR* 3′UTR, over 100 kb away. A physical association between the promoter and the 3′ UTR was detected, suggesting a DNA looping mechanism was involved.[31]

DNA methylation, on the other hand, does not appear to be affected by RNAa[26]—however, this observation may be biased. As it was reported very early on that hypermethylated DNA sequences were poor targets for saRNAs, most saRNAs have been designed to avoid CpG islands and other regions often susceptible to methylation. Nonetheless, the levels of sporadic DNA methylation around which functional saRNAs have been designed are not known to change with gene induction.[26]

The diverse histone modifications associated with RNAa suggests that a number of protein factors may work together with the small RNAs to induce gene activation. Once again, Argonaute

proteins proved to be strong candidates; they have been shown to localize to the nucleus and interact with chromatin, RNA polymerase, epigenetic factors, and a myriad of RNA molecules.[37,38] In Drosophila, in vivo staining revealed that AGO2 was associated at hundreds of sites on polytene chromosomes, mainly in euchromatic (transcriptionally active) regions.[39] Moreover, RNA immunoprecipitation followed by deep sequencing revealed that AGO2-bound small RNAs were enriched in those encompassing promoter regions of many genes.[39] Intriguingly, a very recent report suggests an analogous finding in human cancer cells, where Ago1 was found to interact with RNAP II at active promoters throughout the genome.[40]

Of the four human Argonautes, Ago1–Ago4, only Ago2 possesses catalytic activity.[38] Nonetheless, all four Argonautes have been implicated in gene silencing. In terms of gene activation, Ago3 and Ago4 are either not required, or play very minor roles.[41] In contrast, the knockdown of Ago2 has been shown to abrogate RNAa in a multitude of independent studies.[26,27,36,41] Ago2 is recruited to the site of saRNA binding, and can associate with either promoter DNA or promoter transcripts.[27,41,42] However, whether Ago2 cleavage activity is required for RNAa is unclear. While some studies have suggested a mechanism whereby saRNAs guide the Ago2-mediated cleavage of antisense transcripts to activate gene expression,[11,43,44] others have reported that the targeted non-coding transcripts are not degraded.[27,31,45] Catalytically inactive Ago2 mutants may be used to better understand the importance of transcript cleavage.

A few other proteins have also turned up in the search for the mechanisms of RNAa. Activation of the *PR* gene has been associated with the reduction of HP1, a typical marker of heterochromatin.[27] Heterogeneous nuclear ribonucleoproteins (hnRNPs) may also contribute to RNAa. Affinity purification of biotinylated *p21* promoter-targeting saRNAs followed by mass spectrometry revealed the co-presence of hnRNPA2/B1.[32] Further, knockdown of hnRNPA2/B1 significantly reduced *p21* gene activation. In another study, *PR*-targeting saRNAs were found to shift the localization of hnRNP-k from promoter DNA to the targeted antisense transcript.[27] Interestingly, a recent report has also implicated hnRNP proteins in long non-coding RNA-mediated gene silencing.[46]

Gene activation by small endogenous RNAs

The term "RNAa" was coined by Li et al.[26] in 2006 and has generally been used only when referring to gene activation by exogenously introduced small RNAs. In fact, an endogenous RNA duplex was shown to transcriptionally upregulate a set of neuronal genes two years earlier. Kuwabara et al.[47] identified a ~20 bp RNA species that corresponded to a DNA element commonly found in the promoters of neuron-specific genes. The introduction of this RNA duplex into neural progenitor cells upregulated many of these genes, and induced neural differentiation.[47] Further, the decreased association of histone deacetylases and DNA methylases at these promoters suggested an epigenetic contribution to the mechanism of gene activation.[47]

A few miRNAs may also be able to activate transcription. microRNA-373 (miR-373) has been shown to induce the expression of E-cadherin and CSDC2 genes, both of which contain miR-373-complementary sites in their promoters.[48] Gene activation was dependent on this sequence complementarity, and involved recruitment of RNA polymerase II (RNAP II) to targeted promoters. In another study, miR-205 was shown to transcriptionally activate IL-24 and IL-32, again through complementary elements in the gene promoters.[35] Transcriptional upregulation was associated with H3K4 di- and tri-methylation. In a third study, miR-744 was found to induce *Cyclin B1* expression by directing H3K4 trimethylation as well as RNAP II recruitment.[49] Furthermore, both Ago1 and biotinylated miR-744 were found to associate with the *Cyclin B1* promoter.[49]

piRNAs too, have been implicated in directing epigenetic activation. In Drosophila, a piRNA/PIWI complex has been shown to direct euchromatic epigenetic modifications and promote transcriptional activity in a subtelomeric region of heterochromatin.[50] More recently, piRNA-guided epigenetic activation and repression was shown to occur throughout the Drosophila genome.[51] In *C. elegans*, it has been suggested that piRNAs are able to detect and preferentially silence transgenic sequences containing foreign DNA.[52-54] Remarkably, in a manner analogous to self vs. non-self recognition in the immune system, transgenes containing endogenous sequences seemed to be protected from silencing.[52,54] This mechanism of "antisilencing" is unknown, but appears to involve both small RNAs and Argonaute proteins.[55]

Gene activation by derepression

One mechanism by which small RNA-mediated gene activation may be achieved is through the degradation of natural antisense transcripts (NATs).[6,56] There are two obvious conditions that need to be met for this to be possible. The first is that a NAT must be expressed at the gene of interest; widespread transcription throughout the genome allows this condition to be quite easily satisfied. The second is that the NAT must have an inhibitory effect on the expression of its cognate sense RNA, and this is true for a number of cases examined.[43,44,57] It then follows that small RNAs that can specifically downregulate the antisense RNA (e.g., via canonical RNAi) will induce the activation of the sense transcript.

The activation of the *PTEN* tumor suppressor gene has recently been shown to occur through such a mechanism of derepression.[44] An RNA species transcribed antisense to a *PTEN* pseudogene was found to epigenetically silence PTEN expression.[44] Upon knockdown of this antisense transcript by siRNAs, *PTEN* transcription was significantly upregulated, accompanied by reduced H3K27me3 at the *PTEN* promoter.[44] Similarly, both siRNA- and gapmer-induced degradation of mouse brain-derived neurotrophic factor (BDNF) antisense transcripts resulted in increased levels of BDNF mRNA and protein.[43] Importantly, these results were recapitulated in vivo, and was again found to involve a decrease in promoter-associated H3K27me3 and EZH2, an H3K27 methylase.[43]

A model whereby gene activation occurs through RNAi-mediated degradation of antisense transcripts offers a good explanation for the involvement of Argonaute proteins in RNAa. However, such a mechanism of derepression is likely not the only mode of gene activation. For instance, the expression of many sense and antisense transcripts are directly correlated;[58] while

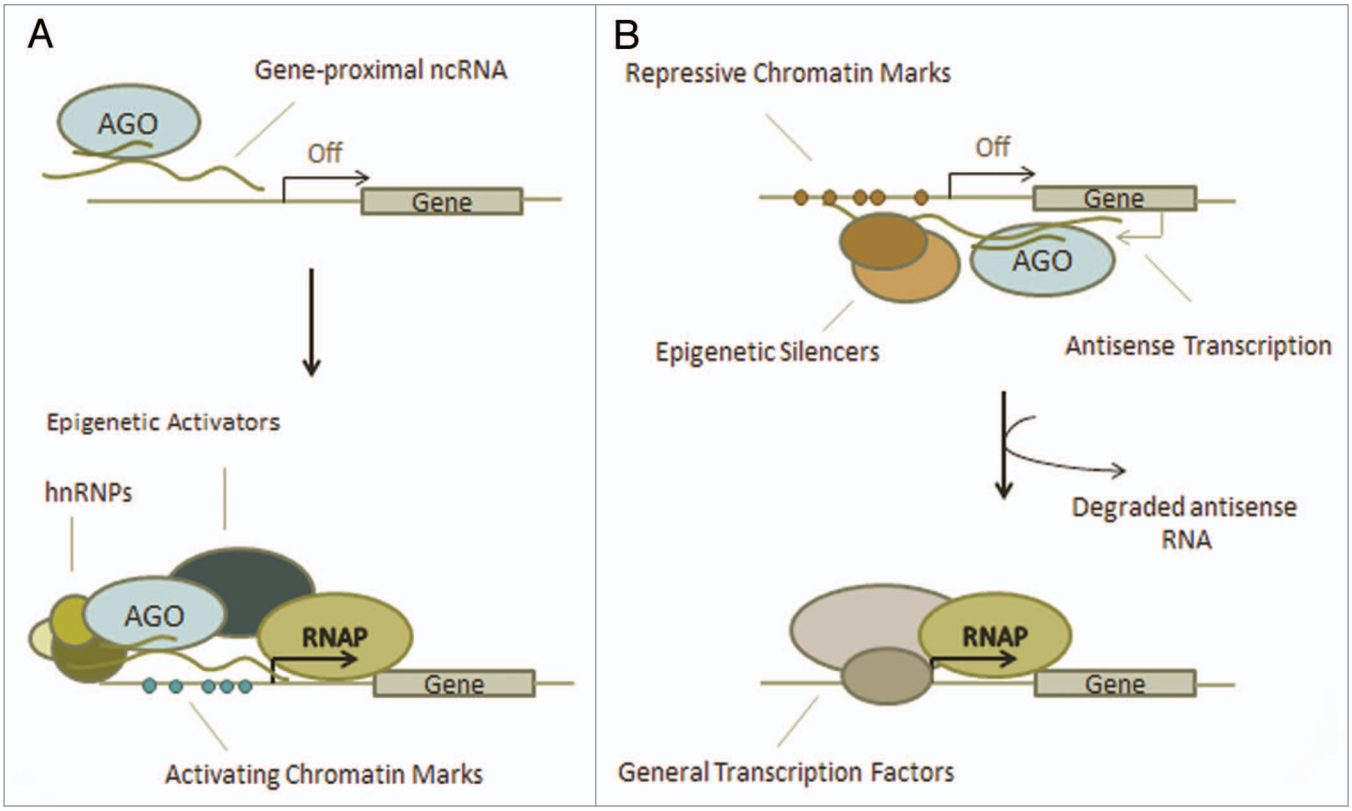

Figure 1. Small RNA-mediated gene activation (**A**) and derepression (**B**). In (**A**), a small RNA (green) targets an Argonaute protein to a promoter-associated ncRNA. This can result in the recruitment of various chromatin modifying complexes and hnRNPs, which together mediate the transcriptional activation of the nearby gene. Importantly, although not shown here, chromosomal looping mechanisms may allow small RNA-Argonaute complexes to regulate promoter activity without directly binding to the promoter region. In (**B**), an antisense transcript recruits epigenetic silencers to the sense gene promoter, repressing sense gene expression. A small RNA-Argonaute complex downregulates the antisense transcript through a classical RNAi pathway, resulting in its degradation and derepressing sense gene expression. Silencing chromatin marks are lost, and the general transcriptional machinery is recruited.

this may be in part due to coordinate regulation by a common element, it suggests that not all antisense transcripts negatively regulate their sense mRNAs. In addition, a number of studies have shown that the binding of an saRNA to a gene-proximal non-coding transcript can activate gene expression without the degradation of the ncRNA.[27,31,45]

Figure 1 illustrates a few ways in which small RNAs may activate gene expression. Although many activating epigenetic changes have been observed at small RNA-targeted loci, there have been few reports identifying the chromatin-modifying complexes responsible. A detailed characterization of nuclear Argonaute-binding partners may help shed light on the elusive mechanisms of small RNA-mediated gene activation.

Gene Activation by Long Non-Coding RNAs

Long non-coding RNAs (lncRNAs) are defined by transcripts longer than ~200 nt with little or no potential to encode functional proteins.[59] Most of the focus to date has been on long intervening non-coding RNAs, or lincRNAs, which do not overlap the exons of known genes, thus simplifying functional interpretations. Unlike some smaller RNAs such as miRNAs, lincRNAs appear to be poorly conserved both in terms of primary sequence and secondary structure.[59] Combined with observations that many lincRNAs are expressed at very low levels, it is reasonable to speculate that a large number are non-functional. Further, in some cases it may be the act of transcription, rather than the non-coding transcript itself, that performs a regulatory role. Nonetheless, it is apparent that at least a subset of identified lincRNAs can carry out important biological functions. In the following section, we summarize the handful of studies that have implicated lincRNAs in the activation of gene expression. Many of these studies have revealed an intimate relationship between lincRNA function and epigenetic regulation.

The Polycomb group (PcG) complexes are a set of conserved epigenetic gene silencers particularly important for the regulation of developmental gene expression.[60] Trithorax group (trxG) complexes antagonize PcG-mediated repression by competing for binding sites in a shared set of target genes.[60] These binding sites, or Polycomb group responsive elements (PREs), are by default bound by PcG complexes, resulting in the silencing of associated genes.[61] In response to certain cellular signals, however, they are displaced by trxG complexes, leading to transcriptional activation.[62] These same DNA elements, when bound by trxG proteins,

are renamed trxG responsive elements (TREs). A series of reports in Drosophila and human cells suggest that the act of transcription through PRE/TREs, together with the ncRNAs produced, serve to signal the switch from PcG repression to trxG activation.

In one study, PRE transcription driven by a constitutive promoter was shown to be sufficient for counteracting PcG-mediated silencing of a reporter gene.[61] Moreover, the insertion of a transcription termination signal abrogated this activation.[61] A function for the non-coding transcripts themselves was tested in a subsequent study, in an endogenous context: upstream of the Drosophila homeotic gene *Ultrabithorax* (*Ubx*), lies a cluster of three transcribed TREs. Ash1, a trxG protein responsible for di- and tri-methylating H3K4, was recruited to the *Ubx* promoter only in cells expressing TRE transcripts.[62] Loss of function studies using siRNAs further showed that these ncRNAs were necessary for gene activation. Interestingly, the transient transfection of TRE transcripts in S2 cells (which lack endogenous TRE transcripts), was sufficient to restore *Ubx* expression.[62] This was also accompanied by recruitment of Ash1 to the *Ubx* promoter, providing an example whereby lncRNAs function in *trans* to direct epigenetic factors to specific genomic loci.

A regulatory module of ncRNAs and histone modification enzymes has also been described at the human homeobox (*Hox*) gene loci. The HOTAIR lincRNA is produced from the *HoxC* locus and acts in *trans* to direct epigenetic silencing of *HoxD* genes.[63] Tsai et al.[64] has shown that HOTAIR acts as a modular scaffold: its 5′ end interacts with Polycomb Repressive Complex 2 (PRC2), which directs H3K27 methylation. Meanwhile, the 3′ end of HOTAIR binds to LSD1, a subunit of the CoREST/REST repressors, capable of demethylating H3K4me2.[64] Thus, HOTAIR is able to direct these complexes to resolve bivalent histone domains at the *HoxD* locus, simultaneously depositing silencing marks while removing activating ones.

Two lincRNAs have been reported to activate transcription at the *HoxA* locus. Wang et al.[65] showed that HOTTIP, a lincRNA transcribed from the 5′ end of the *HoxA* cluster, directs the epigenetic activation of the *HoxA* genes. Interestingly, gene activation occurs in a genomic distance-dependent manner, such that upon knockdown of HOTTIP, genes immediately downstream are more downregulated than those further away. HOTTIP is required to maintain a broad domain of H3K4me3 across the *HoxA* locus, but seems to function in *cis* as its ectopic expression was insufficient to activate a silent *HoxA* locus.[65]

Mistral, another ncRNA shown to be required for the epigenetic activation of *Hoxa6* and *Hoxa7*, lies about 50 kb downstream of *HOTTIP*.[66] Both HOTTIP and Mistral were found to interact with the transcriptional co-activator MLL1, an H3K4 methyltransferase and mammalian homolog of Drosophila Trithorax.[66] Chromosome conformation capture (3C) suggested that retinoic acid-induced differentiation of mouse embryonic stem cells resulted in physical interactions between the *Mistral* gene, *Hoxa6*, and *Hoxa7*.[66] Intrachromosomal rearrangements have also been implicated in gene activation by HOTTIP and DBE-T lincRNAs.[65,67]

The DBE-T lincRNA has been implicated in the pathology of Facioscaphulohumeral muscular dystrophy, a human disease caused by the inappropriate activation of *4q35* genes.[67] A reduction in the copy number of D4Z4 repeats at the *4q35* locus results in the loss of PcG-mediated repression and the expression of the DBE-T lncRNA. DBE-T was found to recruit the Trithorax group protein Ash1L, driving H3K36 dimethylation and the epigenetic activation of the *4q35* locus.[67]

LincRNAs are also known to activate immune response genes. The NeST lncRNA has been shown to activate *Interferon-gamma* (*IFNg*) gene transcription in vivo.[68] NeST RNA interacted with WRD5, a core subunit of MLL complexes, consistent with a correlation between NeST expression and H3K4me3 at the *IFNg* locus. The ectopic expression of NeST RNA was sufficient to induce IFNg synthesis in activated T cells, again suggesting a *trans*-acting mechanism.[68] In another study, a recently identified ncRNA, lincRNA-Cox2, was shown to both activate and repress hundreds of genes upon immune stimulation.[46] LincRNA-Cox2 localized to both the cytoplasm and the nucleus, and associated with hnRNPA2/B1 to mediate its transcriptional repressive effects.[46] It is interesting to note, as previously mentioned, that hnRNPA2/B1 has also been implicated in the transcriptional upregulation by small RNAs.

In 2010, Orom et al.[69] characterized a few thousand ncRNAs using the GENCODE annotation of the human genome. Similar to protein-coding genes, actively transcribed lncRNAs tended to display H3K4me3 at their 5′ ends and H3K36me3 in the gene body. They tested the function of 12 of these lncRNAs using siRNAs against each individual transcript. Interestingly, seven out of 12 lncRNA knockdowns resulted in the concomitant decrease of neighboring genes. When cloned into a heterologous reporter construct, several of these ncRNAs significantly upregulated reporter expression.[69] However, the transgenic overexpression of one specific ncRNA (ncRNA-a7) did not activate its endogenous neighbors, suggesting—at least for this case—a *cis*-mediated mechanism.

In a subsequent study by the same group, stable cell lines expressing the ncRNA-a7-containing reporter construct were used to screen for factors required for the observed RNA-dependent activation. Upon testing a series of known transcriptional activators, it was found that many subunits of the Mediator complex were required specifically for the ncRNA-a7-induced activation of the reporter.[70] ncRNA-a7 directly associated with Mediator, which in turn promoted the association of ncRNA-a7 with target genes through intrachromosomal looping. In vitro assays also showed a ncRNA-dependent stimulation of H3S10 phosphorylation activity of the Mediator complex's CDK8 module, a histone mark associated with transcriptional activation.[70]

Figure 2 shows a simple model whereby a lincRNA may coordinate gene activation, acting as a bridge between targeted chromatin and histone modifying enzymes. While a nascent lincRNA may recruit the chromatin-modifying machinery to genomic regions in its immediate vicinity, it is unclear how *trans*-targeting is specified. In many cases there are no long stretches of sequence complementarity between the lincRNA and the target locus, suggesting that a combination of structural interactions and partial base-pairing may be involved.

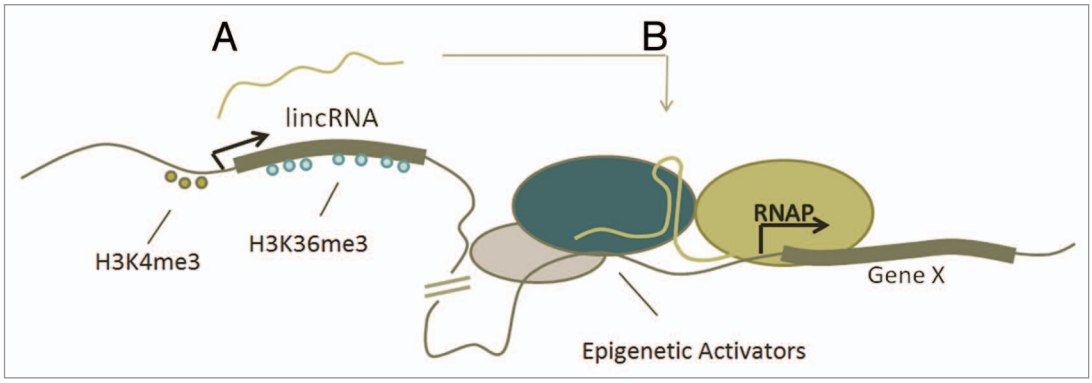

Figure 2. LincRNA-mediated gene activation. (**A**) Transcription of a lincRNA gene with a typical chromatin signature, consisting of an H3K4 trimethylated promoter and a H3K36 trimethylated gene body. (**B**) The lincRNA may act in *cis* or in *trans*, to recruit epigenetic activators to specific genomic regions. Locus specificity may arise from partial complementarity to the lincRNA primary sequence, whereas recruitment of the protein machinery may be more dependent on secondary RNA structures.

Enhancer RNAs

Some of the ncRNAs described in the previous examples display certain characteristics of enhancer sequences. Enhancers are classically defined as DNA elements capable of upregulating gene transcription at variable distances from promoters, independently of orientation.[71,72] Indeed, some of the lincRNAs discussed above upregulated reporter gene expression regardless of the direction in which the ncRNA was cloned. The recent finding that numerous enhancer sequences are also transcribed has created some ambiguities when distinguishing between lincRNAs and enhancer-derived RNAs, or eRNAs. One useful distinction is the typical chromatin signature associated with each transcription unit. Similar to protein coding genes, lincRNAs typically have an H3K4 trimethylated promoter region, and an H3K36 trimethylated gene body.[69,72] In contrast, active enhancers are defined by a high ratio of H3K4me1 to H3K4me3, as well as high levels of acetylated H3K27.[73-75] eRNA transcripts also seem to differ from lincRNAs, the latter often being spliced and polyadenylated.[59]

In 2010, two pioneering studies showed that cell stimulation resulted in the widespread transcription of enhancer regions. In one report, thousands of enhancer-derived transcripts were found to be induced upon macrophage stimulation by endoxin.[76] These non-coding transcripts originated from regions of high H3K4me1 and low H3K4me3, and their induction often preceded the expression of adjacent downstream genes. Furthermore, they were found to be largely unspliced, polyadenylated, strand-specific, and very unstable.[76] A second group reported that membrane depolarization of neurons led to the recruitment of the general transcriptional activator CBP to ~12 000 extragenic regions with enhancer-like chromatin signatures.[77] Six of seven of these regions conferred transcriptional activation when cloned into a luciferase reporter. This activation was independent of sequence orientation but required a functional luciferase promoter, confirming that the identified regions possessed typical enhancer properties. RNA-Seq showed that these regions were transcribed in an activation-dependent manner.[77] However, in contrast to the previous study,[76] the eRNAs generated were mostly bidirectional and unpolyadenylated.[77]

Subsequent reports have suggested that this latter observation represents the more common case. ChIP-seq data for major transcription factors, including p53 and ligand-bound androgen and estrogen receptors, have revealed thousands of binding peaks at extragenic, enhancer-like regions throughout the human genome.[78-81] Transcription factor binding was associated with the bidirectional production of eRNAs with approximate sizes ranging from 1–5 kb.[78-80] qPCR signals from oligo(dT)-primed cDNA were either weak or absent, suggesting that the majority of eRNAs may not be polyadenylated.[78,80]

eRNA production is often correlated with proximal gene induction.[82] Whether these enhancer-derived ncRNAs play a functional role in gene activation, however, has only very recently been examined. As the underlying chromatin is relatively open at enhancers, it seems reasonable that transcripts may be generated simply as byproducts of RNAP II and transcription factor binding. Nonetheless, emerging evidence from both gain- and loss-of-function studies suggests that eRNAs themselves do indeed play a role in transcriptional activation. eRNA knockdown has been achieved by both siRNAs, via the RNAi pathway, and antisense oligonucleotides, via an RNaseH pathway; the downregulation of eRNAs by either approach has been shown to inhibit the induction of nearby mRNAs.[78,81,83]

Gain-of-function studies of eRNA activity have been described using molecular tethering assays. Bringing an eRNA from a specific p53-bound enhancer region into physical proximity with a promoter was shown to be sufficient for transcriptional activation.[78] In another recent study, Li et al.[81] elegantly demonstrated a direct, gene-activating function for a FOXC1 eRNA at the *FOXC1* promoter. They showed that the full, bidirectional *FOXC1* enhancer inserted 2 kb upstream of a *FOXC1* promoter upregulated promoter activity. This upregulation was completely abrogated upon deletion of the sense half of the *FOXC1* enhancer sequence (essentially removing the promoter-proximal production of the sense eRNA). Finally, transcriptional upregulation was fully restored upon tethering of the sense FOXC1 eRNA to the half-enhancer site.[81]

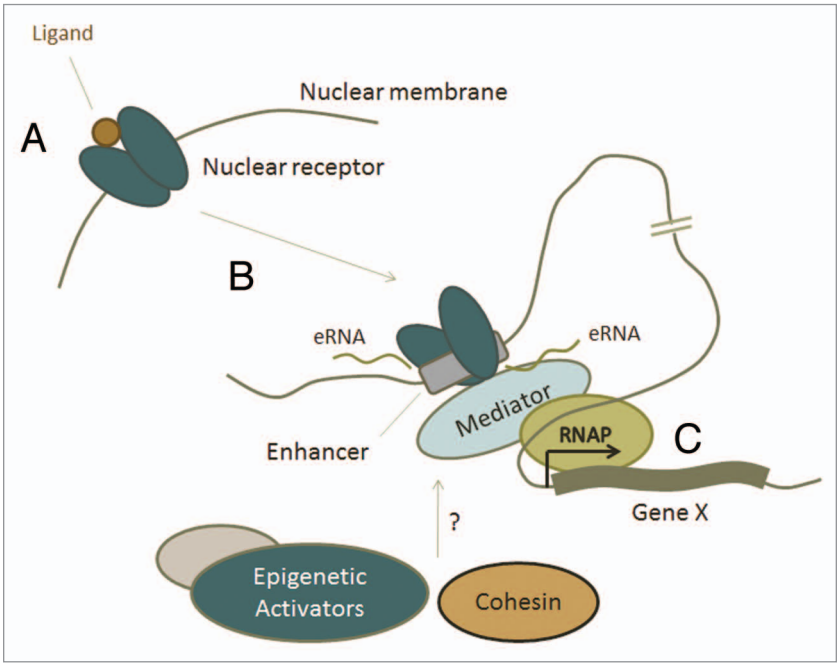

Figure 3. eRNA-mediated gene activation. (**A**) In the example shown here, eRNA-mediated gene activation occurs following a ligand binding to a nuclear receptor. (**B**) The activated receptor dissociates from the membrane and acts as a transcription factor, where it binds to an enhancer region and induces the production of bidirectional eRNAs. (**C**) These eRNAs may lead to increased intrachromosomal interactions with distal promoters, possibly through the Mediator and Cohesin protein complexes. The physical proximity of the enhancer region and/or eRNAs to targeted promoters leads to transcriptional upregulation.

A natural mechanism of tethering eRNAs to their target promoters may be accomplished through DNA looping events. Indeed, various modifications on chromosome conformation capture technologies have confirmed multiple enhancer-promoter interactions.[78,80,81] Some of these interactions may be constitutive, while others are only induced upon cell stimulation. For instance, 17b-estradiol treatment of MCF-7 cells leads to the nuclear localization of the estrogen receptor at thousands of enhancers, inducing both eRNA production and enhancer-promoter looping.[81] Furthermore, these interactions were found to be dependent on eRNAs. Two other protein complexes, cohesin and Mediator, have also been implicated in directing enhancer-promoter looping.[70,79,81] **Figure 3** illustrates an example of eRNA production upon ligand stimulation, leading to transcriptional activation of a downstream gene.

The global binding of transcription factors to enhancer elements can induce cell-specific gene expression; other factors may do the same to mediate specific programs of repression. Rev-Erb-α/β are nuclear receptors that typically act as gene silencers through recruitment of nuclear co-repressors and histone deacetylases.[83] Recently, Rev-Erbs were found to bind to many enhancers selected by macrophage lineage-determination factors. Interestingly, Rev-Erb binding was associated with decreased levels of eRNAs, leading to reduced expression of nearby genes.[83] This loss of enhancer activity was also seen upon eRNA knockdown.[83]

Central Themes of RNA-Mediated Gene Activation

saRNAs, lincRNAs, and eRNAs seem to upregulate gene expression through a number of different mechanisms, many of which are still lacking molecular details. Nonetheless, common themes have emerged, both among gene activating RNAs, and between gene activating and gene silencing RNAs. Two major concepts are that of the RNA guide, and the RNA scaffold.[7] Examples of small RNA guides include piRNAs, which can direct the deposition of activating and repressive epigenetic marks at specific genomic loci.[10,50,51] Similarly, saRNAs and miRNAs have been shown to guide Argonautes to target promoters and epigenetically activate transcription.[33] However, as Argonautes bind ubiquitously to short RNAs, the question remains how various regulatory factors differentially associate with Argonaute complexes to mediate either gene activation or repression. In a sense, small RNAs also act as a scaffold, bringing Argonaute and its various binding partners to sites of complementarity throughout the genome.

Similarly, lincRNAs can act as a binding platform for a number of epigenetic modifiers. As nascent transcripts, lincRNAs would thus guide chromatin modifications in *cis*. As *trans*-acting factors, ncRNAs could possibly direct epigenetic complexes to specific loci through base-pairing with nascent transcripts, by forming RNA:DNA duplexes in unwound regions, or even through RNA:DNA triplexes.[84] It is interesting to consider that, given the current knowledge of the regulatory roles of lincRNAs, there is no requirement that they actually be non-coding. Most lincRNAs are capped, spliced, and polyadenylated, and are thus molecularly identical to protein-coding mRNAs. Therefore, there should be no reason why an mRNA, prior to nuclear export (or via nuclear import) cannot mediate epigenetic regulatory roles as well. In fact, a handful of RNAs with dual coding and non-coding functions have already been described (reviewed by Ulveling et al.[85]).

Another common theme is the correlation of expression between lincRNAs/eRNAs and their neighboring genes. The extent of which this is due to transcriptional "rippling" effects[4] vs. bona fide ncRNA regulatory functions is still debatable, though contributions from both mechanisms are likely. Caution should be taken when interpreting loss-of-function studies by siRNA-mediated knockdown of ncRNAs. Because the introduced siRNAs can themselves direct epigenetic silencing around the target chromatin, knocking down the ncRNAs in question through an RNase H pathway may produce more reliable results.

Even though the exact contribution of ncRNAs to transcriptional activation is unclear, the physical presence of an RNA molecule at particular genomic loci seems capable of mediating diverse regulatory roles. One of these may be to induce chromatin

looping, facilitating interactions between promoters and distally bound transcription factors. The knockdown of eRNAs was shown to reduce enhancer-promoter interactions, suggesting a direct role for eRNAs in gene looping.[81] The HOTTIP lincRNA and a few saRNA target sites have also been shown to be involved in intrachromosomal interactions; however, gain- and loss-of-function studies in these cases suggest that the chromosome configuration is preformed, and independent of the ncRNAs.[31,45,65]

Finally, given the heterogeneity of ncRNA species, it should be expected that mechanisms of RNA-mediated gene activation are not limited to an epigenetic/transcriptional level. For instance, a few miRNAs have been shown to act as posttranscriptional activators of gene expression. For example, miR-369-3 was shown to translationally upregulate TNF-α in a cell-cycle-dependent manner.[86] Similarly, binding of miR-466l to the *IL-10* 3′UTR masks the binding site of an RNA binding protein that would otherwise induce *IL-10* mRNA degradation.[87] In a third example, the let-7 microRNA in *C. elegans* was shown to bind to its own pri-miRNA and upregulate downstream processing events, forming a positive autoregulatory loop.[88] This last example shows, unsurprisingly, that gene activation by ncRNAs is not restricted to protein-coding genes. Moreover, as previously suggested,[6] RNAi-mediated repression of ncRNAs may also result in the upregulation of reciprocally regulated genes.

As the majority of miRNAs still appear to act as silencers of gene expression, another way to activate—or derepress—mRNA expression would be to employ transcripts that can compete for miRNA binding. This hypothesis was proposed by Salmena et al.,[89] whereby competing endogenous RNAs (ceRNAs) regulate each other through competition for a limited pool of miRNAs. Because individual miRNAs have many predicted targets, and individual transcripts often harbor many miRNA binding sites, the ceRNA hypothesis suggests a massive, interconnected regulatory network that involves all types of RNA transcripts.[90]

Concluding Remarks

Given that many of the reports discussed in this review are very recent, it appears that we have barely begun to appreciate the tremendous diversity of ncRNAs, and how they may impact gene expression. The sheer number of potentially functional transcripts, from small 20 nt RNAs to 10 kb lincRNAs, will likely be reflected in their equally diverse mechanisms of action. Cytoplasmic ncRNAs can modulate gene expression through binding to complementary mRNAs, whereas in the nucleus, they may interact with chromosomal DNA, as well as the myriad of coding and non-coding transcripts. The complexity of RNA-mediated gene regulatory networks that will undoubtedly be unveiled in the near future is difficult to imagine. Perhaps terms such as "RNA-mediated gene regulation" will no longer be used, as it will have an even broader meaning than "protein-mediated gene regulation."

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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